DOI:
10.1016/j.thromres.2018.07.015

Document Version
Peer reviewed version

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PII: S0049-3848(18)30423-7
DOI: doi:10.1016/j.thromres.2018.07.015
Reference: TR 7093

To appear in: *Thrombosis Research*

Received date: 24 May 2018
Revised date: 30 June 2018
Accepted date: 9 July 2018

Please cite this article as: S.P. Grover, P. Saha, J. Humphries, O.T. Lyons, A.S. Patel, J. Serneels, B. Modarai, M. Mazzone, A. Smith , Inhibition of prolyl hydroxylase domain proteins selectively enhances venous thrombus neovascularisation. Tr (2018), doi:10.1016/j.thromres.2018.07.015

Inhibition of prolyl hydroxylase domain proteins selectively enhances venous thrombus neovascularisation

Running Title: PHDs regulate venous thrombus neovascularisation

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Abstract

BACKGROUND: Hypoxia within acute venous thrombi is thought to drive resolution through stabilisation of hypoxia inducible factor 1 alpha (HIF1α). Prolyl hydroxylase domain (PHD) isoforms are critical regulators of HIF1α stability. Non-selective inhibition of PHD isoforms with l-mimosine has been shown to increase HIF1α stabilisation and promote thrombus resolution.
OBJECTIVE: The aim of this study was to investigate the therapeutic potential of PHD inhibition in venous thrombus resolution.
METHODS: Thrombosis was induced in the inferior vena cava of mice using a combination of flow restriction and endothelial activation. Gene and protein expression of PHD isoforms in the resolving thrombus was measured by RT-PCR and immunohistochemistry. Thrombus resolution was quantified in mice treated with pan PHD inhibitors AKB-4924 and JNJ-42041935 or inducible all-cell *Phd2* knockouts by micro-computed tomography, 3D high frequency ultrasound or endpoint histology.
RESULTS: Resolving venous thrombi demonstrated significant temporal gene expression profiles for PHD2 and PHD3 ($P<0.05$), but not for PHD1. PHD isoform protein expression was localised to early and late inflammatory cell infiltrates. Treatment with selective pan PHD inhibitors, AKB-4924 and JNJ-42041935, enhanced thrombus neovascularisation ($P<0.05$), but had no significant effect on overall thrombus resolution. Thrombus resolution or its markers, macrophage accumulation and neovascularisation, did not differ significantly in inducible all-cell homozygous *Phd2* knockouts compared with littermate controls ($P>0.05$).
CONCLUSIONS: This data suggests that PHD-mediated thrombus neovascularisation has a limited role in the resolution of venous thrombi. Directly targeting angiogenesis alone may not be a viable therapeutic strategy to enhance venous thrombus resolution.

Key words
Venous thrombosis, tissue remodelling, hypoxia, animal models

Word count: 3248

Introduction

Deep vein thrombosis (DVT) is a common condition with an annual incidence of roughly 1 in 1000 in the general population [1]. Common complications include pulmonary embolism and post-thrombotic syndrome (PTS) that are significant sources of mortality and morbidity respectively [2]. Clinical studies show that rapid clearance of thrombus, by thrombolysis or faster natural resolution, is associated with a reduced incidence of PTS [3, 4]. While effective in preventing recurrence standard anticoagulant therapies do not aid in clearance of the initial thrombotic insult. Novel treatments that aim to enhance natural venous thrombus resolution may, therefore, be of value in reducing the burden of PTS in patients with DVT.

The early venous thrombus is acutely hypoxic resulting in nuclear accumulation of HIF1α in thrombus resident cells [5]. Enhanced stabilisation of HIF1α in the thrombus following treatment with l-mimosine significantly enhanced thrombus resolution, accompanied by increased macrophage recruitment, neovascular channel formation and the production of vascular endothelial growth factor A (VEGF-A) [5]. Expression of HIF1α and the transcriptional target VEGF-A are also increased in the thrombosed vessel wall [6]. In contrast to l-mimosine adenoviral overexpression of HIF1α in the thrombus failed to significantly enhance thrombus resolution despite resulting in a doubling of active HIF1α [7]. HIF2α is also expressed in the resolving venous thrombus primarily in macrophage rich regions, however, the contribution of this isoform to thrombus resolution has yet to be determined [8].

The biological effect of l-mimosine has been attributed to the capacity of this agent to inhibit the prolyl hydroxylase domain (PHD) containing proteins. However, it is important to note that as a potent metal ion chelator this agent may also inhibit the activity of related enzymes including the collagen prolyl 4-hydroxylases [9-11]. In a recent study Kiriakidis and colleagues demonstrated that l-mimosine inhibited the collagen prolyl-4-hydroxylase dependent release of complement C1q by macrophages [12].

PHD proteins serve as critical molecular oxygen sensors through regulation of HIFα subunit stability. Under-normoxic conditions PHD isoforms hydroxylate HIFα subunits in an oxygen and 2-oxoglutarate dependent manner leading to ubiquitination and subsequent proteasomal degradation. Under hypoxic conditions in which oxygen is rate-limiting PHD isoforms are inactivated allowing HIFα subunits to translocate to the nucleus and form transcriptionally active complexes [13]. HIF1α transcriptional targets include a number of genes implicated in thrombus resolution including VEGF-A, plasminogen activator inhibitor 1 and inducible nitric oxide synthase[14].

Recently a number of novel PHD inhibitors have been developed, which are reported to offer improved specificity[15, 16]. The capacity of this new generation of PHD inhibitors to modulate venous thrombus resolution has yet to be determined. In parallel, the capacity of individual PHD isoforms to regulate cellular responses to tissue hypoxia has been extensively studied using isoform specific knockout mice. Importantly, these studies have strongly implicated loss of PHD2 with enhanced macrophage recruitment and

angiogenesis, processes implicated in the resolution of venous thrombi [17-19]. However, the role of PHD2 in venous thrombus resolution has yet to be determined.

The aim of this study was to determine the expression and distribution of PHD isoforms in the resolving thrombus. The role of PHD isoforms in thrombus resolution was investigated using the PHD inhibitors AKB-4924 and JNJ-42041935. To complement the small molecule inhibitor strategy thrombus resolution was also characterised in inducible all-cell *Phd2* knockouts.

Methods

PHD2 Knockouts

For studies of inducible *Phd2* deletion *Phd2*$^{fl/fl}$; Rosa26creERT2/+ mice were crossed with *Phd2*$^{fl/+}$ to obtain mice with the following genotypes (effective genotype in parentheses): *Phd2*$^{fl/fl}$ (*Phd2*$^{+/+}$), *Phd2*$^{fl/+}$ (*Phd2*$^{+/+}$), *Phd2*$^{fl/+}$;R26creERT2/+ (*Phd2*$^{+/-}$) and *Phd2*$^{fl/fl}$;R26creERT2/+ (*Phd2*$^{-/-}$) as previously described [20]. Conditional gene deletion was induced by intraperitoneal injection of Tamoxifen (Sigma, USA) at a dose of 1mg/mouse/day, for 5 days prior to thrombus induction [20]. Demographics of knockout mice and data of thrombus formation efficiency can be found in the data supplement (Supplementary Tables S1).

Model of venous thrombosis

All experimental animal procedures were carried out in accordance with the Animal (Scientific Procedures) Act 1986 (King's College London, UK) or approved by the Institutional Animal Care and Research Advisory Committee (K.U. Leuven, Belgium). For inducible *Phd2* knockout studies 6-12 week-old male and female mice on a C57BL/6J background were used. For natural thrombus resolution and PHD inhibitor studies 8-10 week old male BALB/c mice were used. Thrombus was induced using the St Thomas' model of inferior vena cava thrombosis as previously described [21, 22]. Mice 6-12 weeks in age were anaesthetised by isoflurane inhalation (3%, 1L/Min O_2). A midline laparotomy was made, the bowel externalised and blunt dissection used to reveal the retroperitoneal structures. Using sharp dissection the IVC was separated from the aorta immediately distal to the left renal vein. A piece of 4-0 Mersilk (Ethicon, USA) suture material was passed behind the IVC and tied onto a length of 5-0 prolene (Ethicon, UK) placed along the vessel. The prolene was removed resulting in an approximate 90% stenosis of the vessel. A mini bulldog serrefine vascular clamp (Fine Scientific Tools, Germany) was applied to the IVC at two locations distal to the site of stenosis for 20 seconds. Perioperative buprenorphine was administered by intraperitoneal injection at a dose of 0.1mg/kg. The bowel was internalised and layered closure of the abdomen achieved with continuous 4-0 polydiaxanone sutures (Ethicon, USA). Induction of thrombus in knockout mice was conducted in an operator-blinded manner.

Drug Dosing

AKB-4924 (kindly provided by Dr Robert Shalwitz, Akebia Therapeutics, USA) was provided at a concentration of 2mg/ml in 40% (v/v) aqueous 2-hydroxypropyl-beta cyclodextrin, 60% (v/v) 50mM citrate buffer pH5. 8-10 week old male BALB/c mice were randomised to receive either AKB-4924 at a dose of 5mg/kg or 10mg/kg, or vehicle control by daily subcutaneous injection starting 24hrs after thrombus induction, based on previous reports in the literature demonstrating doses in this range stabilized HIF1α and reduced disease severity in a model of colitis [15, 23, 24]. JNJ-42041935 (kindly provided by Michael Rabinowitz, Johnson and Johnson, USA) was prepared in 20% (v/v) 2-hydroxypropyl-beta cyclodextrin at a concentration of 3mg/ml. 8-10 week old male BALB/c mice were randomised to receive either JNJ-42041935 at a dose of 35mg/kg or vehicle control by daily intraperitoneal injection starting 24 hours after thrombus induction based on previous reports in the literature demonstrating this dose is sufficient to induce HIF1α mediated erythropoietin expression and increase haemotocrit [16, 25, 26].

Imaging of thrombus resolution

Histology IVC containing thrombus was excised, fixed overnight in 10% (v/v) formal saline and processed for paraffin embedding. Transverse sections (5μm) were cut at 500μm intervals though the sample generating a series of levels over the entire length of the thrombus. Brightfield images of haematoxylin and eosin stained thrombus were captured and measurement of thrombus cross sectional area obtained (Image Pro Plus software, Media Cybernetics, UK) to enable analysis of thrombus volume and vein lumen recanalisation as previously described [5, 21].

Contrast-enhanced microCT Thrombus was imaged by contrast-enhanced microCT as previously described [27]. Images were reconstructed using VivoQuant software (v1.22, Invicro, USA) at a voxel size of 65μm. Reconstructed scans were segmented and analysed using ITKsnap software (Open Source) by a blinded observer to provide measurements of thrombus volume (see supplemental methods).

3D high frequency ultrasound Thrombus was visualised by 3D HFUS using a Vevo2100 imaging unit with a 40MHz ultrasound probe attached to a stepper motor (Visual Sonics, Canada). A more detailed description is provided in the supplementary methods. Images were exported as DICOM files and thrombus segmented using Osirix software (v5.5, Open Source) by a blinded observer to provide measurements of thrombus volume (see supplemental methods) [28].

Expression and localisation of PHDs

Thrombus qPCR Thrombi formed in 8-10 week old male Balb/c mice were separated from the surrounding IVC, immersed in RNAlater (Thermo Fisher), snap frozen in liquid nitrogen and stored at -80°C. Total RNA (mRNA) was extracted using Trizol (Thermo Fisher) and the RNeasy mini kit (Qiagen, Germany). RNA was reverse transcribed using the high-capacity RNA-to-cDNA kit (Applied Biosystems, USA) and cDNA for *phds1-3* quantified. Expression of PHD isoforms was normalised against housekeeping genes, *Actb* and *Gak* (see supplemental methods).

Immunohistochemistry (IHC) Expression of PHD1, PHD2 and PHD3 was localised in the naturally resolving venous thrombus by IHC. After inhibition or deletion of PHD2 thrombus resident Mac2 positive macrophages and CD31 positive endothelial channels were localised by IHC. Further details of antigen retrieval, primary antibodies, signal amplification and subsequent analysis are provided in the supplemental methods. Images were obtained at 50x and 200x with a light microscope (DMRB, Leica, Germany) using a digital camera (Micropublisher 3.3, QImaging, Canada). Stained sections were compared to appropriate IgG control to ensure specificity. Measurements of staining were obtained using Image Pro Plus software (Media Cybernetics, UK).

Haematocrit measurements
Blood was drawn under terminal anaesthesia by cardiocentesis into graduated EDTA anti-coagulated tubes (Greiner Bio-One, UK), centrifuged for 10mins at 10,0000xg with measures of total volume of blood collected and mean corpuscular volume used to estimate haematocrit.

Statistics
Normality of data was assessed using D'Agostino and Pearson omnibus tests with parametric and non-parametric statistics selected as appropriate. Details of statistical tests used for individual datasets are provided in the accompanying figure legends. Data are represented as mean ± standard error (SE). Results were analysed using Prism software (v5, GraphPad, USA) with $P<0.05$ considered significant.

Results

Expression of PHD isoforms during natural thrombus resolution
Expression of *Phd1, 2* and *3* genes was detected at days 1, 3, 7 and 11 post-induction by qPCR. While expression of *Phd1* did not significantly change over time ($P>0.05$, Fig 1a), expression of *Phd2* and *Phd3* was found to change significantly over the timecourse of thrombus resolution studied ($P<0.05$, Fig 1b-c). Localisation of PHD1, 2 and 3 protein by immunohistochemistry revealed expression in the acute venous thrombus that may be attributed to the polymorphonuclear infiltrate whilst expression in the chronic thrombus is most likely associated with mononuclear cells that predominate at later time-points (Fig 1d).

Effect of pan PHD inhibition, using AKB-4924 and JNJ-42041935 PHD inhibitors, on venous thrombus resolution
Thrombus resolution was measured by longitudinal contrast-enhanced microCT at days 1, 7 and 14 post-induction in mice treated with the PHD inhibitor AKB-4924. There was no significant effect on temporal changes in thrombus size or rate of resolution following treatment with either 5 or 10mg/kg AKB-4924 ($P>0.05$, Fig 2a-c). Similarly, endpoint histological measurements of thrombus volume and vein lumen recanalisation did not differ significantly after AKB-4924 treatment ($P>0.05$, Fig 2d-e). A dose

dependent increase in thrombus macrophage content was observed at day 14, but this was not significant (P>0.05, Fig 2f). However, treatment with AKB-4924 was associated with a significant increase in thrombus neovascularisation (P<0.001, Fig 2g) attributed to the 10mg/kg treatment group (P<0.05).

In mice treated with the PHD inhibitor JNJ-42041935 thrombus resolution was quantified by end-point histology at day 14 post-induction. Resolution, as determined by measurements of thrombus volume, vein lumen recanalisation and macrophage accumulation, was unchanged (P>0.05, Fig 3a-c). However, neovascular channel formation, at day 14 post-induction, was significantly enhanced by treatment with this inhibitor (P<0.05, Fig 3d). Treatment with JNJ-42041935 also resulted in a robust increase in haematocrit consistent with effective inhibition of PHD isoforms (P<0.01, Fig S1).

Thrombus resolution in all-cell inducible *Phd2* knockouts

Thrombus resolution in all-cell inducible *Phd2* knockouts was assessed longitudinally by 3D HFUS (Fig 4a). Thrombus volume in heterozygous and homozygous inducible *Phd2* knockouts at days 1, 7 and 14 post-induction did not differ significantly from wild-type littermate controls (P>0.05 Fig 4b). To take into account differences in thrombus size at the time of formation rates of thrombus resolution were also determined. The rate of resolution between days 1-7 and 7-14 was similarly unaffected by deletion of phd2 (P>0.05, Fig 4c). Vein lumen recanalisation, thrombus macrophage content and thrombus neovascularisation were unaltered in mice deficient for *Phd2* when measured at day 14 post-induction (P>0.05, Fig 4d-f). Deletion of *Phd2* was assessed in cytoplasmic preparations of kidney extracted at the time of thrombus collection. A significant reduction in the levels of PHD2 protein expression, but not PHD3, was observed in mice with a homozygous deficiency for *Phd2* (Fig S2). Efficient deletion was further supported by significantly increased haematocrit measurements observed at day 14 post-induction in *Phd2* homozygous knockouts compared with wild-type littermate controls (P<0.001, Fig 4g).

Discussion

Thrombus resolution occurs through an organisational process of tissue remodelling, hallmarks of which include the recruitment of macrophages and the development of neovascular channels. We have previously suggested that the development of these channels is important for resolution [29] and may be enhanced by promoting HIF1α accumulation [5]. PHD mediated regulation of HIF1α stability may, therefore, represent an important mechanism in the resolution of venous thrombi. This study investigates the effect of manipulating the expression and activity of PHD enzymes on thrombus resolution.

Expression of all PHD isoforms was detected at both the transcriptional and protein level in the resolving thrombus. Temporal analysis of *Phd* gene expression revealed a significant increase in *Phd2* gene expression as the

thrombus resolved. It is likely that these changes in expression can be attributed to the changing cellular composition of the thrombus as it resolves. PHD protein expression appears localised to inflammatory cells that accumulate within the thrombus. At acute time-points PHD protein expression was observed in polymorphonuclear cells, most likely neutrophils that predominate in the thrombus at this time [30]. At later time-points PHD expression was observed in mononucleated cells, most likely macrophages recruited to the resolving thrombus [30, 31]. It is, however, possible that at later time-points PHD isoforms are expressed in fibroblasts [32].

Expression of PHD isoforms in both macrophages and neutrophils is consistent with recent observations. Neutrophils have been shown to express all three PHD isoforms with loss of *Phd3* resulting in impaired survival of neutrophil under hypoxic challenge [33]. PHD isoforms are considered important regulators of macrophage polarisation [34]. Whereas loss of *Phd2* results in skewing of macrophages towards an alternatively activated, pro-remodelling state [35] macrophages deficient in *Phd3* demonstrate an enhanced pro-inflammatory potential [36, 37].

Given the observed expression of multiple PHD proteins in the resolving venous thrombus initial efforts focused on the effect of broad pharmacological PHD inhibition. The PHD inhibitors AKB-4924 [15], a metal ion chelator and JNJ-42041935 [16], a 2-oxoglutarate mimetic, were administered to mice and the effect on thrombus resolution determined. Treatment with either of these agents did not accelerate resolution, however, a significant (50%) increase in neovascular channel formation was observed at 14 days post-induction. The pro-angiogenic effect of these agents is consistent with *in vitro* studies that demonstrate the capacity of AKB-4924 to stabilise HIFα subunits and induce expression of the transcriptional target *Vegfa* [38, 39]. This observation is also supported by numerous *in vitro* studies in which classical PHD inhibtors were found to enhance angiogenesis, providing evidence for inhibitor activity in our model [40-42]. Despite an established role for PHD isoforms in regulating macrophage function we did not observe a significant difference in recruitment of this cell type or evidence of differential remodelling of collagen (data not shown).

Deletion of *Phd2* confers an improved response to ischaemic challenge in a variety of cell types [19, 20, 35]. An inducible strategy was selected because of the embryonic lethality of constitutive homozygous *Phd2* deletion. *Phd2* was deleted in all-cells in a tamoxifen dependent manner using the inducible Rosa26creERT2 line crossed onto the *Phd2* floxed line. Tamoxifen was administered to mice for 5days immediately prior to induction of thrombus formation. It is important to note that a recent study has indicated that tamoxifen is an inhibitor of platelet activation and angiogenic potential and this may have altered thrombus resolution, however, all mice received tamoxifen and so this is likely controlled for in the experimental design [43]. Deletion of *Phd2* was confirmed by a significant reduction in renal PHD2 protein expression in inducible homozygous knockouts. This finding was further supported by significantly increased haematocrit observed in inducible homozygous *Phd2* knockouts consistent with induction of the HIF target

erythropoietin [44]. Elevated haematocrit has previously been reported in PHD deficient mice and supports effective deletion in this study [45, 46].

The proportion of mice that formed thrombi did not differ significantly based on *Phd2* genotype, however, a slight non-significant decrease in induction efficiency was noted in Phd2-/- mice compared to wild-type littermate controls (see supplementary Table 1). This study was not formally powered to determine the effect of *Phd2* gene deletion on thrombus formation. It is interesting to consider whether hypoxic signalling may be involved in the pathogenesis in venous thrombus formation. Additional studies of Phd2 gene knockouts and pre-treatment with selective PHD inhibitors prior to thrombus induction may reveal a role for this family of enzymes in venous thrombus formation.

Similar to studies of mice treated with PHD inhibitors, no significant differences in measures of thrombus resolution were observed in inducible *Phd2* knockout mice. Unlike inhibitor treated mice, however, thrombus neovascularisation was largely unaffected by loss of *Phd2* expression. As demonstrated by our gene expression and immunohistochemical staining both PHD1 and PHD3 are also expressed in the resolving thrombus. It is likely that in *Phd2* knockouts expression of additional PHD isoforms compensate for loss of PHD2 activity. By contrast in studies using pan-PHD inhibitors all three isoforms are inhibited leading to a more pronounced upregulation of HIFα-mediated angiogenic gene expression. Alternatively, it is possible that PHD1 and PHD3 act as the primary regulators of angiogenic gene expression and subsequent thrombus resolution neovascularization. Future studies should investigate whether deletion of PHD1 or PHD3 is sufficient to promote thrombus neovascularization.

It is possible that the observed increases in haematocrit after PHD inhibition or *Phd2* gene deletion may have masked the expected pro-remodelling phenotype. Elevated haematocrit has been shown to increase platelet adhesion and thrombus formation under conditions of high shear [47]. Recent work has further supported the prothrombotic potential of elevated haematocrit in a ferric chloride model of arterial thrombosis [48]. Computational modelling further suggested that elevated haematocrit causes increased margination of platelets that may facilitate increased adhesion at sites of vascular injury [48]. The contribution of changes in haematocrit to venous thrombus formation is, however, unclear. In our current study despite significantly elevated haematocrit we did not observe an increase in thrombus size at day 1 in inducible homozygous *Phd2* knockouts suggesting a minimal contribution of haematocrit in this setting.

The failure of AKB-4924 and JNJ-42041935 to accelerate venous thrombus resolution is in contrast to our previous observations with l-mimosine [6]. As a potent ion chelator it is possible that l-mimosine inhibits targets, other than the PHD enzymes, that contribute to enhanced thrombus resolution [9-12]. Further experiments are required to establish the mechanism of action by which l-mimosine accelerates venous thrombus resolution. The results of the present study more closely agree with our experience overexpressing constitutively active HIF1α in the acute venous thrombus. Despite a significant increase in

levels of active HIF1α only a minor (20%), non-significant reduction in thrombus volume was observed at day 7 post-induction [7]. Importantly, this genetic approach was not subject to the same potential for off-target effects as treatment with pharmacological inhibitors. Taken together with the current inhibitor studies these data would suggest that HIF1α plays a more limited role in thrombus resolution than previously hypothesised.

During natural resolution the venous thrombus is permeated by a network of endothelial lined neovascular channels[29]. The contribution of thrombus neovascularisation to subsequent resolution, however, remains unclear, with conflicting data reported. Treatment with pro-angiogenic basic fibroblast growth factor significantly enhanced thrombus neovascularisation independent of changes in thrombus size in a rat model of IVC ligation[49]. Although treatment of thrombus with direct administration of an adenoviral VEGF-A gene construct resulted in a remarkable enhancement of thrombus resolution and vein recanalisation, this was mainly associated with macrophage infiltration rather than neovascular channel formation[50]. This study adds to the body of work suggesting that venous thrombus resolution is driven by processes other than neovascularisation that likely include macrophage-mediated tissue remodelling and fibrinolysis. The contribution of angiogenic factor signalling to thrombus resolution should not be discounted, however, as resolution is delayed in endothelial specific *Vegfr2* knockouts [51].

The results of this study lead us to conclude that broad-inhibition of PHD enzymes selectively enhances venous thrombus neovascularisation, but that this is largely independent of resolution. This suggests that direct targeting of angiogenesis alone may not be a viable therapeutic strategy for enhancing venous thrombus resolution.

Acknowledgements

This study was funded by a non-clinical PhD studentship from the British Heart Foundation to SG (FS/10/51/28677). The PET/CT platform at King's College London was funded by an equipment grant from the Wellcome Trust (WT 084052/Z/07/Z). The authors thank Stephen Clark for assistance with contrast-enhanced microCT imaging and Stéphanie De Vleeschauwer for assistance in establishing the 3D HFUS imaging protocol.

Author Contributions

S.P.G., P.S., J.H. and J.S. designed and conducted experiments. O.T.L. and A.S.P. assisted with data analysis and interpretation. S.P.G and A.S. wrote the manuscript. B.M. and M.M. interpreted data and edited the manuscript. A.S. supervised the entire study. All authors approved the final version of the manuscript.

Additional Information

The authors declare no competing financial interests.

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Figures

Figure 1: Expression of PHD isoforms in the naturally resolving venous thrombus
PHD gene and protein expression was assessed in thrombi formed in 8-10 week old male Balb/c mice. Gene expression of (a) *Phd1*, (b) *Phd2*, and (c) *Phd3* was quantified by qPCR at days 1, 3, 7 and 11 post-induction (1-way ANOVA with post-hoc Bonferroni, n=5 per group) and expressed as normalised fluorescence intensity. (d) IHC localisation of PHD isoforms (black staining) expressing cells were localised to the naturally resolving venous thrombus at days 3 and 14 post-induction (n=4 per group).

Figure 2: Effect of the PHD inhibitor AKB-4924 on venous thrombus resolution
(a) Representative images of IVC resident thrombus visualised by contrast-enhanced microCT at days 1, 7 and 14 post-induction after treatment with 5mg/kg AKB-4924, 10mg/kg AKB-4924 or vehicle control (scale bar = 2mm). Contrast-enhanced microCT derived measurements of (b) thrombus volume or (c) rate of thrombus volume were unchanged by treatments (2-way and 1-way ANOVA respectively $P>0.05$, n=6 per group). Endpoint histological measurements at 14days post induction of (d) thrombus volume, (e) vein lumen recanalisation and (f) macrophage recruitment were also unchanged (1-way ANOVA, $P>0.05$). (g) A significant increase in the number of CD31 positive neovascular channels was observed after treatment with AKB-4924 (1-way ANOVA $P<0.001$). Data represented as mean ± SE. *Post-hoc Bonferroni $P<0.05$ 10mg/kg AKB-4924 vs vehicle.

Figure 3: Effect of the PHD inhibitor JNJ-42041935 on venous thrombus resolution
Histological measurements of (a) thrombus volume, (b) vein lumen recanalisation, (c) macrophage content and (d) thrombus neovascularisation at day 14 post-induction after treatment with 35mg/kg JNJ-42041935 were compared to vehicle control (students t-test, n= 9 per group). Data represented as mean ± SE.

Figure 4: Effect of inducible *Phd2* gene deletion on venous thrombus resolution
(a) Representative transverse slices and 3D reconstructions of IVC resident thrombus (red dotted line) adjacent to the aorta (white arrowhead) visualised by 3D HFUS at days 1, 7 and 14 post-induction in wild-type, (scale bars = 2mm). Measurements of (b) thrombus volume and (c) the rate of thrombus resolution derived from 3D HFUS were unchanged in $Phd2^{fl/fl}$ and $Phd2^{fl/+}$ ($Phd2^{+/+}$), $Phd2^{fl/+}$;R26creERT2/+ ($Phd2^{+/-}$) and $Phd2^{fl/fl}$;R26creERT2/+ ($Phd2^{-/-}$) mice (2-way and 1-way ANOVA respectively, n=5-7 per group). End-point histological measurements, at 14 days post induction, of (d) vein lumen recanalisation, (e) macrophage content and (f) neovascularisation were also unchanged (1-way ANOVA). (g) End-point measurements of haematocrit were significantly increased in inducible homozygous *Phd2* knockouts compared to wild-type littermate controls (1-way ANOVA). * Post-hoc Bonferroni $P<0.05$ -/- vs +/+. Data represented as mean ± SE.

Highlights

- Tissue hypoxia is thought to be a critical driver of venous thrombus resolution
- Prolyl hydroxylase domain proteins (PHDs) are critical oxygen sensors
- Broad pharmacological PHD inhibition selectively enhances neovascularisation
- Thrombus neovascularisation and resolution occur independent of *Phd2* gene deletion

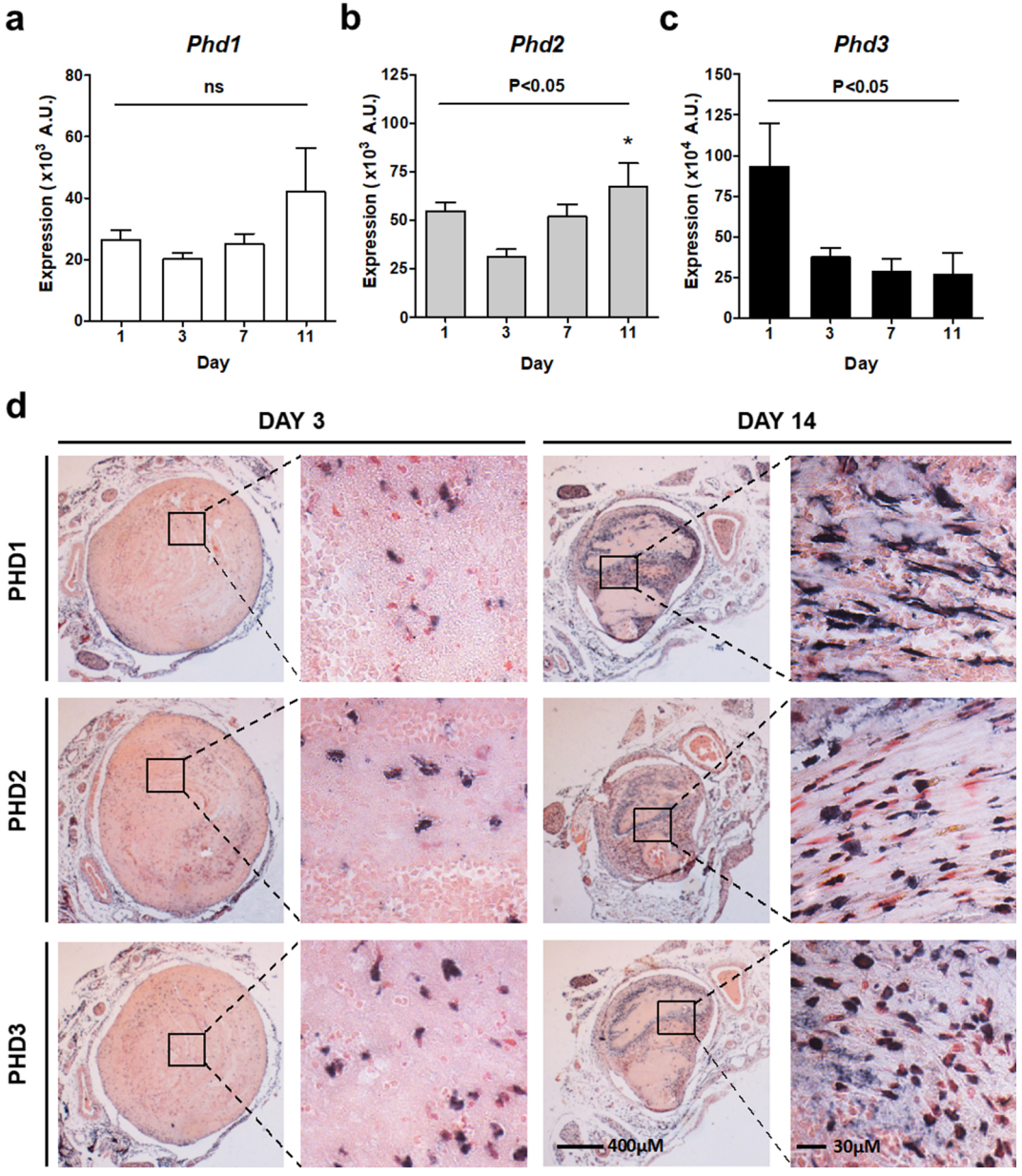
a
Phd1
ns
Expression (x10³ A.U.)
80
60
40
20
0
1
3
7
11
Day
b
Phd2
P<0.05
*
Expression (x10³ A.U.)
125
100
75
50
25
0
1
3
7
11
Day
c
Phd3
P<0.05
Expression (x10⁴ A.U.)
150
125
100
75
50
25
0
1
3
7
11
Day
d
DAY 3
DAY 14
PHD1
PHD2
PHD3
400µM
30µM

Figure 1

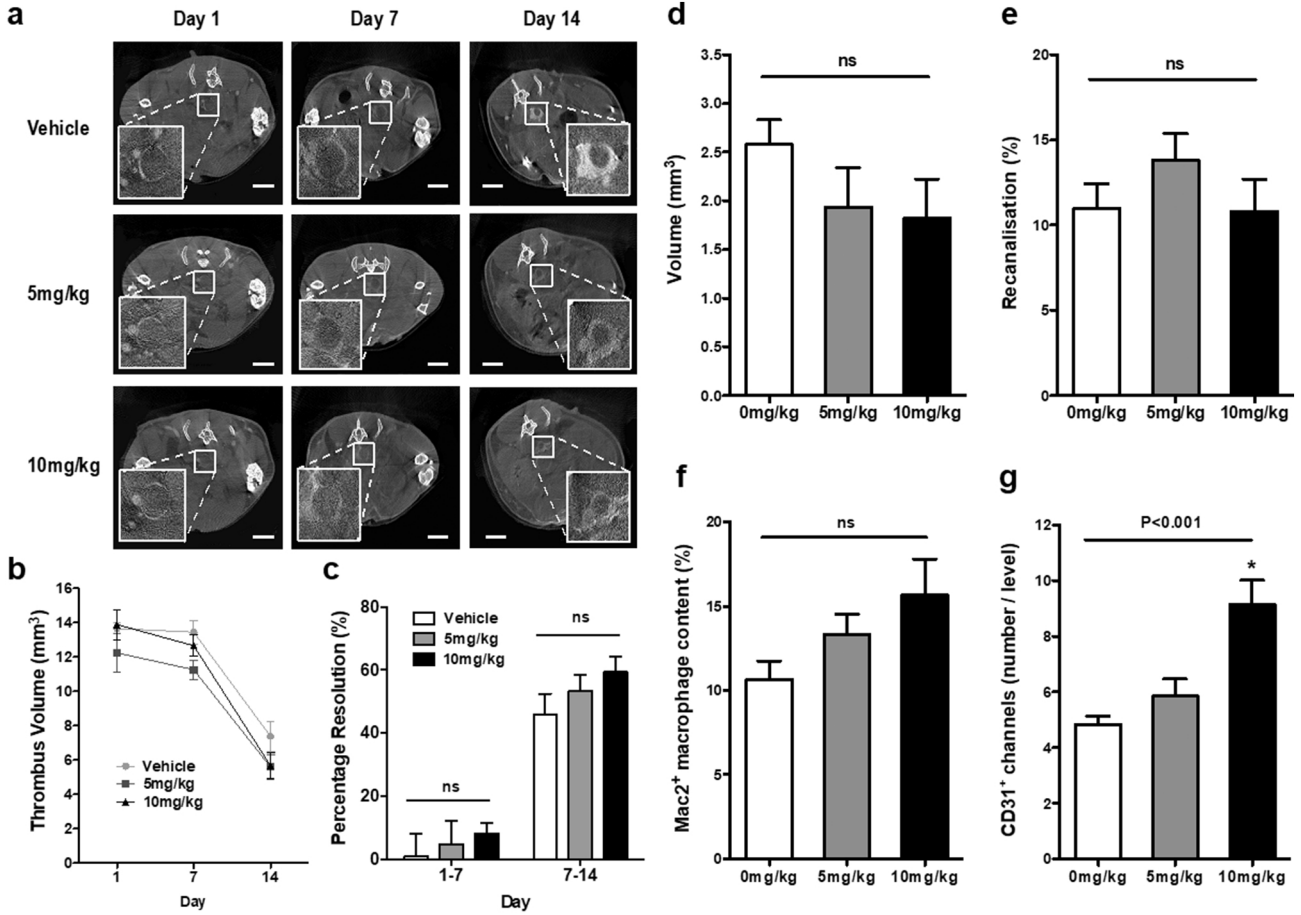
a
Day 1
Day 7
Day 14
Vehicle
5mg/kg
10mg/kg
b
Thrombus Volume (mm3)
Vehicle
5mg/kg
10mg/kg
1
7
14
Day
c
Percentage Resolution (%)
Vehicle
5mg/kg
10mg/kg
ns
ns
1-7
7-14
Day
d
Volume (mm3)
ns
0mg/kg
5mg/kg
10mg/kg
e
Recanalisation (%)
ns
0mg/kg
5mg/kg
10mg/kg
f
Mac2+ macrophage content (%)
ns
0mg/kg
5mg/kg
10mg/kg
g
CD31+ channels (number / level)
P<0.001
*
0mg/kg
5mg/kg
10mg/kg

Figure 2

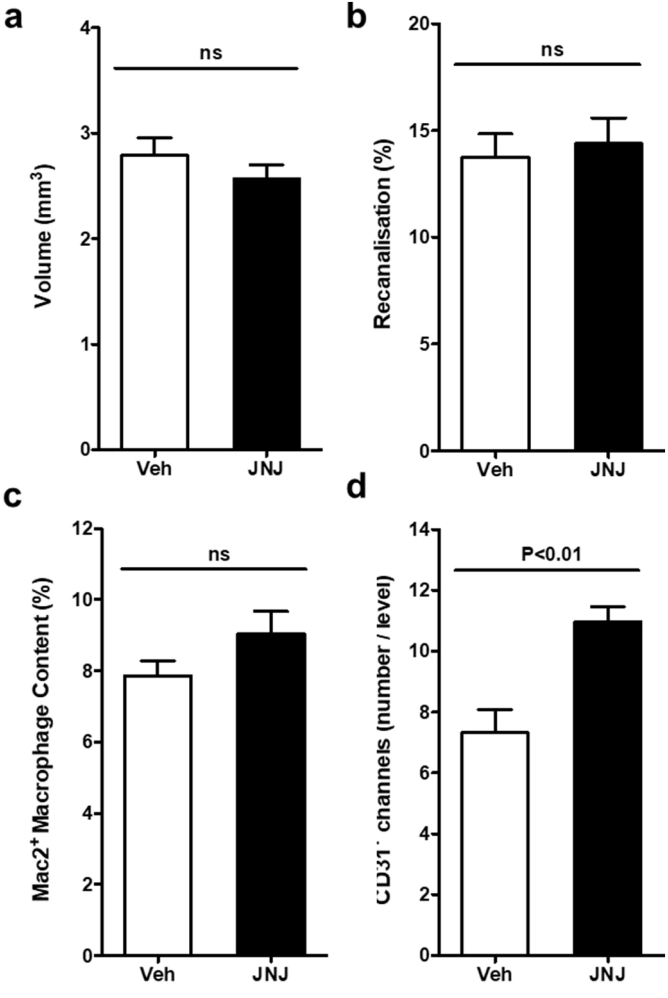
a
Volume (mm3)
ns
Veh
JNJ
b
Recanalisation (%)
ns
Veh
JNJ
c
Mac2+ Macrophage Content (%)
ns
Veh
JNJ
d
CD31+ channels (number / level)
P<0.01
Veh
JNJ

Figure 3

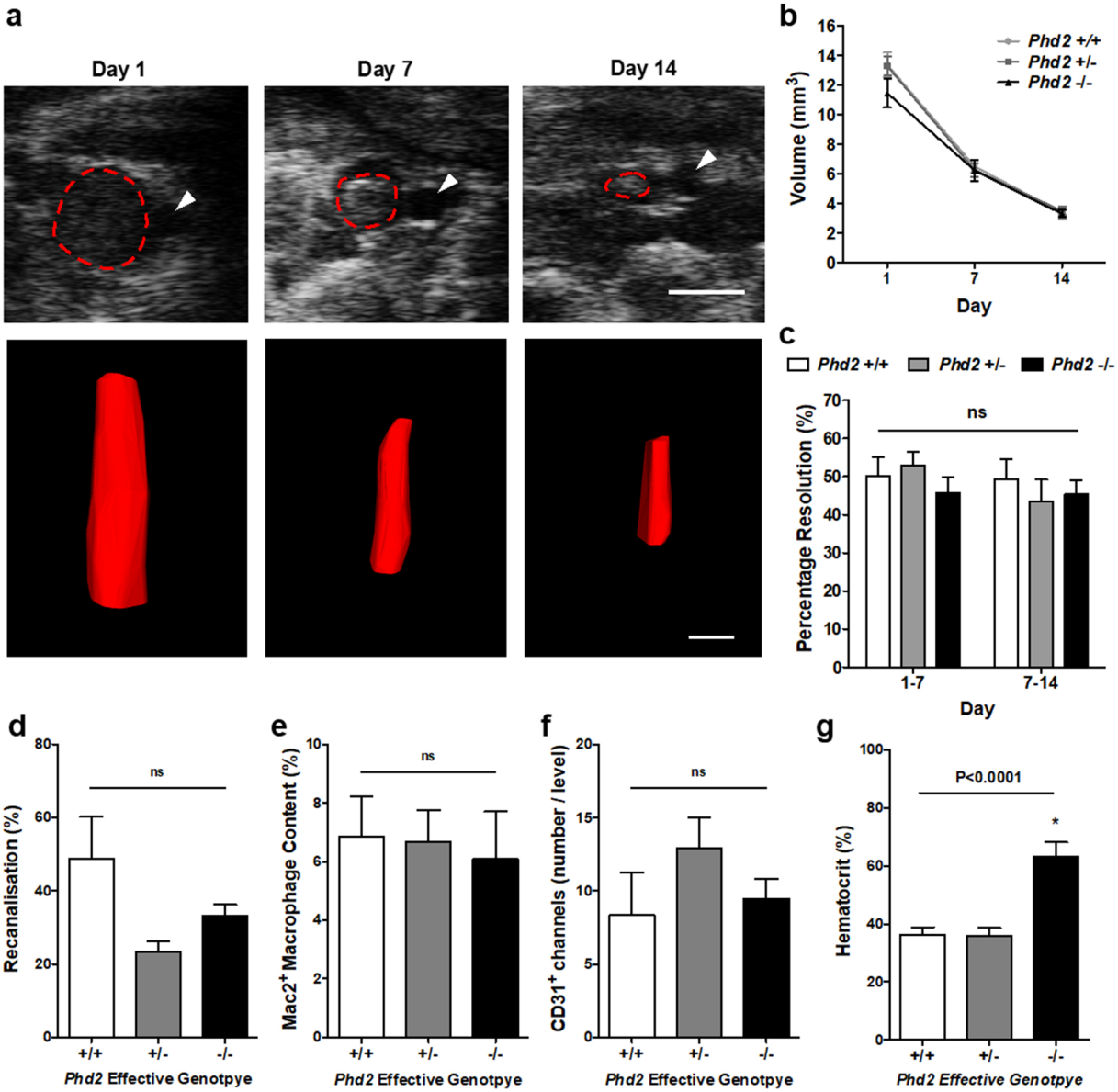
a
Day 1
Day 7
Day 14
b
Phd2 +/+
Phd2 +/-
Phd2 -/-
Volume (mm3)
Day
c
Phd2 +/+
Phd2 +/-
Phd2 -/-
ns
Percentage Resolution (%)
1-7
7-14
Day
d
ns
Recanalisation (%)
+/+
+/-
-/-
Phd2 Effective Genotpye
e
ns
Mac2+ Macrophage Content (%)
Phd2 Effective Genotpye
f
ns
CD31+ channels (number / level)
Phd2 Effective Genotpye
g
P<0.0001
*
Hematocrit (%)
Phd2 Effective Genotpye

Figure 4